Protein Trafficking in Kinetoplastid Protozoa

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INTRODUCTION

The Kinetoplastid protozoa encompass a wide range of monogenetic and digenetic species, which infect hosts ranging from invertebrates to plants and mammals (223). They are the earliest-branching organisms in eucaryotic evolution to have either mitochondria or peroxisomes (114). It is therefore of considerable interest to investigate their mechanisms of protein trafficking, because the results enable us to identify characteristics that have truly been conserved throughout eucaryotic evolution and may therefore have been present in the hypothetical progenitor of all higher eucaryotic organisms. Most studies have concentrated on species of medical or economic importance. These are *Trypanosoma cruzi* (the agent of

Chagas' disease), various *Leishmania* species (the agents of Oriental sore, espundia, and kala-azar); and the salivarian or African trypanosomes (the agents of sleeping sickness and nagana).

ULTRASTRUCTURE

A generalized diagram of a kinetoplastid is shown in Fig. 1. There are considerable variations between different species and even between different life cycle stages within a species; forms are classified according to the overall dimensions, flagellar morphology, and position of the kinetoplast (mitochondrial DNA) and flagellar basal body relative to the nucleus (Fig. 2) (223). For example, promastigotes, epimastigotes, and trypomastigotes are all long, spindle-shaped cells, but in trypomastigotes the flagellum runs along most of the cell body. Amastigotes are roughly spherical with only a flagellar remnant. The shape is maintained by a corset of closely arrayed microtubules

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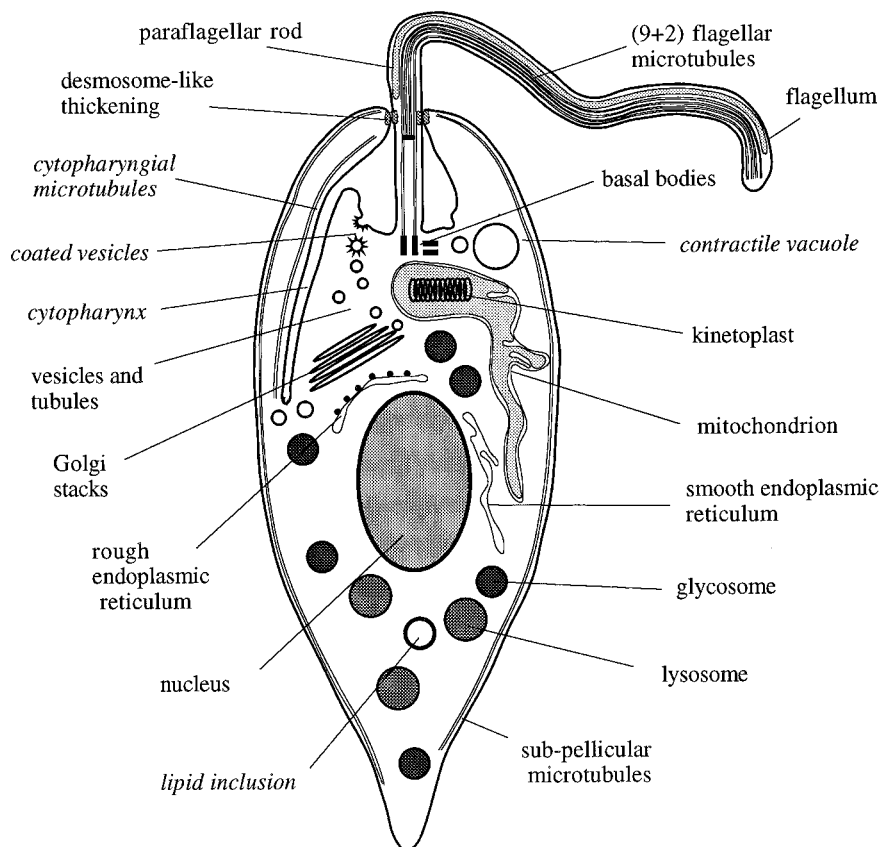
FIG. 1. Generalized diagram of a kinetoplastid protozoan. Structures that are found in only some species or life cycle stages are labelled in italics. The diagram is not to scale.

(244) that spiral around the cell immediately beneath the plasma membrane; in trypomastigotes, their "+" ends are near the point of flagellar exit from the cell (173). The flagellum arises from a specialized invagination called the flagellar pocket (233). It is anchored in the cell by a basal body which is physically linked to the mitochondrion. The complex network of mitochondrial DNA, the kinetoplast, is concentrated in this part of the mitochondrion. A partially developed basal body lies perpendicular to the major one and will mature at the next round of cell division; both basal bodies take part in kinetoplast division (172). In free-living kinetoplastids, the second basal body functions to anchor a second flagellum (224). Near the flagellar pocket entrance, the cortical microtubules bend inward and appear to dive into the parasite in parallel with the cell membrane, gradually becoming thinner until they peter out entirely where the plasma membrane comes into contact with the flagellum (27, 224). A small number of specialized microtubules (usually four) dives down into the pocket (27, 57, 138). In forms with an externally attached flagellum, the attachment to the cell body is seen as an increase in electron density which interrupts the cortical microtubule array (224). (The "undulating membrane" seen next to the flagellum by light microscopy is really just a pulled-out portion of the plasma membrane.) Immediately next to the flagellar attachment are the same four microtubules that extend to the base of the flagellar pocket; they are readily distinguished because they are always associated with smooth endoplasmic reticulum (188). Desmosome-like thickenings are present where flagella attach to each other, to insect epithelia, or to debris (26, 197).

They are a common feature of all insect-form trypanosomatids (138).

The cortical microtubules represent an efficient barrier against vesicular transport (224). The need to protect the bulk of the surface from the environment also means that essential receptors must be exposed on a privileged part of the plasma membrane. In forms lacking a cytopharynx (see below), all endocytosis and exocytosis occurs at the flagellar pocket (199, 233; see reference 110 and references therein). The entrance to the flagellar pocket is guarded by desmosome-like tight junctions between the membranes of the cell itself and the flagellum (26). In electron micrographs, the desmosomes do not form a continuous barrier, and it is clear that large molecules such as ferritin and antibodies can move both in and out. If or how this movement is restricted and what role is played by flagellar beating motions are unknown; perhaps the movement of the flagellum opens and closes the desmosomes, or perhaps channels between the desmosomes are continuously present. Pinocytic vesicles can be seen in all trypanosomatids; vesicles with a coated morphology are not seen in insect stages, being restricted to a few life cycle stages such as bloodstream forms of salivarian trypanosomes.

In addition to the flagellar pocket, some members of the family have a separate specialized organelle, the cytopharynx. This is a funnel- or cleft-shaped invagination extending toward the posterior of the cell. Bodonids are free-living organisms that feed on bacteria; they have two morphologically and functionally distinct flagellae. The bodonid cytopharynx, presumed to be used for ingesting bacteria, is a long membrane-bound
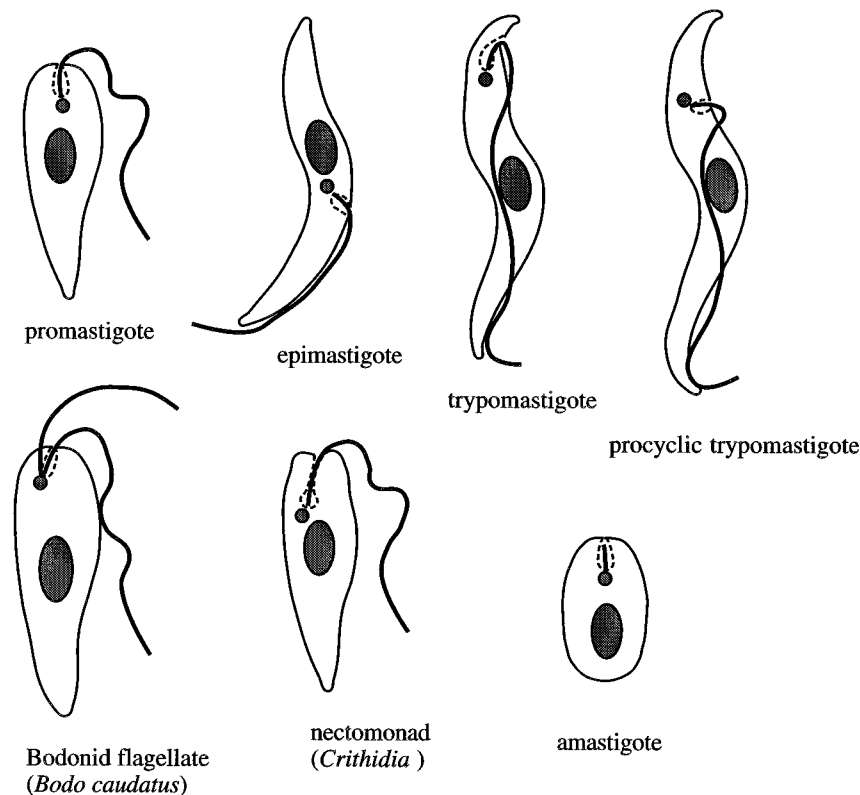
FIG. 2. Various forms of kinetoplastid protozoa. The diagram is not to scale and is drastically simplified from reference 223, where more details can be found.

tube opening quite near (but not in) the flagellar pocket and supported by a "rod organ" of triangular cross section composed of 15 microtubules (224). The cytopharynx of *T. mega* (224), *T. raiae* (164), and *T. cruzi* epimastigotes and amastigotes (136, 138, 190) also opens near the point where the flagellum emerges; that of *T. cruzi* epimastigotes contains amorphous material that stains with a carbohydrate stain (50). In *Crithidia* species and *T. conorhini* (136), the cytopharynx opens into the base of the flagellar pocket. In all cases, the cytopharynx is accompanied by four or more microtubules, doubtless evolutionarily related to the ones that accompany the flagellum along the plasma membrane in (cytopharynxless) trypomastigotes. In *Crithidia* species, these four microtubules extend their path along the flagellar pocket past the basal body toward the nucleus; two go even further toward the posterior of the cell. Between the Golgi apparatus and the flagellar pocket, these microtubules are associated with a complex assemblage of tubules and vesicles; where this assemblage abuts the pocket, there is an indentation of the pocket membrane (cytostome) that appears denser than the surrounding membrane and is presumably a site of particularly active vesicle fusion, pinocytosis, or budding (27).

Whether the vesicles and tubules of the cytopharynx are involved in exocytosis, endocytosis, or both is unclear. Although ferritin has been observed at the bottom of the cytostome and also in vesicles nearby, it is not clear that the vesicles were actually derived from the base of the cytostome, because it generally terminates in the posterior of the cell, where lysosomes are also found (27, 50, 164). In general, far more vesicles are clustered near the flagellar pocket than near the cytostome. The ultrastructural observations could clearly form a basis for new functional studies, because almost nothing is known about

what the cytopharynx and accompanying microtubules do. A possible scenario is that the "original" function of the cytopharynx in an ancestral free-living kinetoplastid was in the uptake and digestion of bacterial prey. If this is so, it must have been lost several times independently during subsequent evolution (68).

Internally, kinetoplastids have a nucleus, rough and smooth endoplasmic reticulum, and (usually) a single morphologically identifiable Golgi apparatus. Endocytic or exocytic structures identified by serial sectioning of *T. brucei* and *T. congolense* included tubulovesicular structures similar to mammalian endosomes (200 nm in diameter), small vesicles and cisternae (20 to 25 nm in diameter), and large (200 to 300 nm in diameter) lysosome-like structures (230). Kinetoplastid lysosomes often lie toward the end of the cell that does not contain the flagellar pocket and vary in size and number according to species and life cycle stage; they may be simple or multivesiculate (138, 230). Distinguishing all these vesicular structures is difficult, as reliable markers are in most cases not available (see below). The mitochondrion can also be relatively simple or highly branched with plate-like or tubular cristae. Glycolysis occurs in microbodies known as glycosomes that are evolutionarily related to peroxisomes. *Crithidia fasciculata* and *Leishmania* species contain a "contractile vacuole" speculated to be involved in osmotic regulation (224), and many types have storage vacuoles, such as stored lipid droplets. Internalized bacteria and bacteroid bodies (endosymbionts) are present in several species, such as members of the *Bodonidae* and *Crithidia* species. A variety of different cell fractionation methods has been used to separate functionally different compartments of trypanosomatids (3, 83, 85, 148, 150, 198).

It is not known how the position of the Golgi apparatus,

between the nucleus and flagellar pocket, is maintained; neither is it known what determines the direction of vesicular transport. Internal to the cortical cytoskeleton, the only cytoplasmic microtubules are those lining the cytopharynx—in those forms that have one. No other cytoplasmic filaments have been found, although actin genes are present and transcribed. It would be interesting to see what effects inhibitors of microtubule motors, or of microtubule assembly and disassembly, have on trypanosomatid vesicular transport or internal organisation (125; see reference 66 for recent references).

LIFE CYCLES AND THE CELL SURFACE

Trypanosomatid cell surfaces are highly specialized to provide protection against a chemically, enzymatically, or immunologically hostile environment. In some pathogenic forms, one or a few molecular species serve to shield the underlying membrane and sometimes play a role in invasion of host cells.

African Trypanosomes

Much more is known about the cell biology of the African (or salivarian) trypanosomes *T. brucei brucei*, *T. brucei rhodesiense*, and *T. congolense* than about that of other kinetoplastids. African trypanosomes live in the blood and tissue fluid of a mammalian host; the bloodstream form trypomastigotes are relatively long (about 20 μm), with a flagellum that emerges from the posterior end (defined relative to the direction of motion) and is attached along the body of the parasite (Fig. 2). The cell surface is protected by a thick layer of variant surface glycoprotein (VSG) which is changed at frequent intervals: this antigenic switching enables the parasite to survive indefinitely in the face of recurrent immune responses (46). Energy metabolism depends on substrate-level phosphorylation, provided by a high rate of glycolysis; the mitochondrion is rudimentary (65). Stumpy, nondividing forms may survive preferentially when the parasites are taken up by a feeding tsetse fly; in the fly midgut, the parasites differentiate into procyclic forms which are morphologically quite similar to bloodstream forms, although the kinetoplast is relatively nearer the nucleus. Procyclic metabolism is very different: an elaborate mitochondrion is capable of oxidative phosphorylation (65), the major substrate is proline, and the VSG coat is replaced by an acidic protein of unknown function (procyclic acidic repetitive protein [PARP] or procyclin in *T. brucei*, glutamic acid-rich protein [GARP] in *T. congolense*) (12, 13, 37, 174). Before entering the mammalian bloodstream, procyclic trypanosomes differentiate into epimastigotes and then into VSG-bearing metacyclic forms in the tsetse fly salivary glands. The major cell surface proteins in African trypanosomes are linked to the plasma membrane via glycosyl phosphatidylinositol (GPI) anchors whose composition varies according to the antigenic variant and life cycle stage (129).

The changing of the VSG coat is mediated by a combination of transcriptional control and genetic rearrangement. The expressed gene is situated near a telomere, at the end of a polycistronic transcription unit up to 70 kb long. Several possible expression sites exist in the genome and can be turned on or off in a mutually exclusive fashion; in addition, the telomeric VSG genes themselves can be exchanged or replaced (reviewed in references 159 and 220). Between the expressed VSG and the promoter lie up to 10 other genes known as expression site-associated genes (ESAGs). These include several with potential membrane-spanning domains, and the gene products of three ESAGs have known function and are directed to the cell surface: ESAG 6 and ESAG 7 encode a transferrin receptor in the flagellar pocket (see below), and ESAG 4 encodes an adenylate cyclase that is distributed over the surface of the flagellum (154).

In addition to VSGs and ESAGs, two invariant bloodstream form-specific surface glycoproteins are found in *T. brucei*. They have molecular masses of 65 and 75 kDa and are accessible to biotinylation (248) and surface iodination (102); however, antibodies can react with them only after the cells have been fixed with formaldehyde (247). Presumably, the VSG shields them from the immune response. The predicted sequences are not related either to each other or to any other known sequence, and each includes a single transmembrane alpha helix, a short C-terminal cytoplasmic domain, and a consensus signal sequence whose cleavage has been shown by N-terminal sequencing of the mature protein (247). No function is known. The presence of various plasma membrane enzymes has been demonstrated (see references 14 and 102 and references therein), but nothing is known about their trafficking. The region of flagellar attachment of trypomastigotes contains specialized glycoproteins (20, 243) identified by monoclonal antibodies.

As mentioned above, during the transformation from bloodstream forms to procyclic forms, the VSG coat is rapidly shed and replaced by PARP. The discovery of GPI-specific phospholipase C (PLC) in trypanosomes raised the tempting possibility that this enzyme was responsible for VSG release. Unfortunately, the enzyme is found on the cytoplasmic face of vesicles—not at the cell surface—so a role in VSG release is rather unlikely (28). Studies of the release indicated that proteolysis of the VSG was involved, but was unaffected by lysomotropic amines, suggesting that the digestion does not occur in an acidic compartment of the cell (29, 249). In addition to PARP and essential transporters, procyclic trypanosomes possess a membrane-bound *trans*-sialidase activity that is able to transfer sialic acid residues to PARP. Its precise location has not been determined, but solubilization with PLD suggested that the protein might bear a lipid anchor (64).

Leishmania Species

Leishmania species are transmitted by sandflies. The insect-living forms—noninfective promastigotes and infective metacyclic promastigotes—are long, slender parasites with a free flagellum. They bear on their surface a GPI-anchored zinc metalloprotease (commonly known as gp63) and are coated with a GPI-anchored carbohydrate, lipophosphoglycan (LPG) (see reference 7 and references within). Differences in either the glycan chain length or composition of LPG distinguish the infective from the noninfective forms. Upon entering the mammal, the infective forms invade macrophages, where they multiply inside lysosomes as amastigote forms; roles for both gp63 and LPG in invasion have been suggested (177). In amastigotes, gp63 is not surface accessible and lacks a GPI anchor (131) and LPG is either strongly down-regulated or absent. Low-molecular-mass glycoinositol-phospholipids are synthesized throughout the life cycle (see references 184 and 240 and references therein). gp63 is encoded by several related but nonidentical genes: the levels of the mRNAs are developmentally regulated (see, for example, references 169 and 171), and their products differ in their posttranslational processing and intracellular targeting (131). The gene class C1 from *Leishmania mexicana* is expressed predominantly in amastigotes. The predicted protein bears an extended hydrophobic C terminus that differs in sequence from that of the major GPI-anchored species, leading to speculation that this sequence is responsible for the different location and processing of gp63 in amastigotes (130).

Infective metacyclics and amastigotes of *L. major* also have at their surface the gene B protein. This 177-residue protein is a member of a developmentally regulated gene family. The predicted protein is hydrophilic and acidic, and 45% of the sequence is predicted to encode 7-amino-acid repeats with limited homology to the cell wall-binding domain of protein A from *Staphylococcus aureus* (70). A cytoplasmically oriented plasma membrane calcium ATPase (126) and a plasma membrane-associated 3′ nucleotidase/nuclease (31) have been characterized from *L. donovani* promastigotes.

T. cruzi

T. cruzi trypomastigotes from the blood are taken up into reduviid bugs, in whose midgut they replicate as epimastigotes. Epimastigotes bear a low-molecular-weight membrane-bound glycoconjugate, lipopeptidophosphoglycan, on their surface (see reference 79 and references therein). Metacyclic trypomastigotes are excreted in the feces and can invade via the wound made by the feeding bug. They attach to and invade host cells, where they escape from the endocytic vacuole into the host cell cytoplasm. The parasites multiply intracellularly as amastigote forms, which escape as the cell lyses, transforming into circulating trypomastigotes which are competent to reinvade fresh host cells or to infect a feeding reduviid bug. Attachment to host cells is mediated by surface *trans*-sialidases (see below) in consort with penetrin (95) and possibly also (for metacyclics) an 82-kDa adhesin (170). Penetrin binds glycosaminoglycans on the target cell surface (95); recombinant penetrin mediates both attachment and invasion when expressed in bacteria (152). Information on the primary structure of penetrin is not yet available, but it would appear (from its surface location) that the signals that mediate export to the cell surface in *T. cruzi* are also functional in *E. coli* (152). Escape from the endocytic vacuole is probably mediated by a pore-forming protein that is secreted by the parasites and activated by endosome acidification (4, 115). The major surface glycoproteins of bloodstream trypomastigotes are encoded by several families of related genes sharing about 30% amino acid sequence identity and some repetitive epitopes (32). One family (trypomastigote surface antigens) encodes proteins of about 85 kDa that bear a consensus sequence for GPI anchor addition. Another family of proteins in the 120- to 200-kDa range includes molecules with neuraminidase and/or *trans*-sialidase activity. Both protein families are GPI anchored (32, 45). Surface proteins specific to metacyclic trypomastigotes have also been reported; these again show homologies to the gp85/sialidase family and are predicted to be GPI anchored (see reference 6 and references therein). The amastigote forms bear stage-specific "amastins" on their surface (211); the proteins can also be detected immunologically in intracellular vesicles. One amastin sequence contains four hydrophobic regions, including one each at the N and C termini, and is in general fairly hydrophobic. This may be the first family of major surface proteins in kinetoplastids that is not held in the membrane via a GPI anchor but, rather, by a classical hydrophobic peptide domain.

The gp72 protein of *T. cruzi* is a surface glycoprotein involved in flagellar attachment. In a "knockout" mutant, the flagellum flies free (39, 48, 143). The gp72 mutant has altered motility, and although there is no marked impairment of its ability to survive in vitro, both development of trypomastigote forms and propagation in the *Triatoma* vector is impaired (39). Nothing is known about how flagellar attachment proteins are targeted to their destination.

Other Trypanosomatids

Studies of the surfaces of other trypanosomatids of lesser (or no) economic or medical importance have been restricted mainly to evolutionary comparisons. Thus, gp63 homologs have been found in the mosquito parasite *C. fasciculata* (101) (where the sequence has a potential GPI anchor addition signal) and a baboon parasite of the species *Endotrypanum* (132); the latter also have *trans*-sialidase activity, but ultrastructural localization has not been done. In addition, a surface 3′-nucleotidase/nuclease has been purified from *C. luciliae* (144). This activity was found to be induced by cultivation in the absence of purines or phosphate; so far, this is the only reproducible example in the kinetoplastids of the induction of an enzyme activity by simple nutrient manipulation (144).

TRANSPORTERS

Various trypanosomatid transporters, responsible for ion homeostasis and the uptake of nutrients, have been identified (reviewed in reference 250), but only a few have been characterized in detail. Glucose transport is of clear interest, because it is likely to be essential for survival of some parasite stages; most results are consistent with a facilitated diffusion mechanism (see reference 214 and references therein). Genes encoding two hexose transporters have been cloned from *T. brucei* (25). One, *THT-1*, is expressed in bloodstream forms. The product of this gene, when expressed in oocytes, had the properties of the major glucose transporter of bloodstream forms. The second gene, *THT-2*, is expressed in procyclic forms; whether it mediates import of glucose into the cells or from the cytoplasm into the glycosomes has not been determined, but the presence of a different transporter for procyclic forms was supported by kinetic data (141). *T. cruzi* also has a hexose transporter gene (214) whose function has been confirmed by oocyte injection; genes that are members of the glucose transporter superfamily have also been isolated from *Leishmania* species (109). Although the locations of all these hexose transporters in the cell have not been defined, they contain multiple membrane-spanning domains and are clearly likely to be integral proteins of either the glycosomal or plasma membrane. Two glucose transporters of *L. enriettii* differ only in their N-terminal hydrophilic domains (163). Immunolocalization of these proteins in normal promastigote parasites or in parasites overexpressing epitope-tagged versions of the putative transporters revealed that one is distributed over the plasma membrane and flagellar pocket whereas the other is found on the surface of the flagellum (163). The N-terminal hydrophilic domain is clearly responsible for this sorting. It is not known whether either or both isoforms follow the normal route of export via the flagellar pocket; the sorting mechanism will be an exciting topic for further investigation.

Many other small molecules must also be transported across trypanosomatid membranes. The mechanism of proline uptake by *Leishmania* species is still controversial (212); procyclic forms of *T. brucei* must also be able to transport proline as it is a major energy source. The members of the *Trypanosomatidae* salvage purines from the host; African trypanosomes possess two distinct adenosine transporters, one of which appears to be responsible for uptake of the arsenical drug melarsen (so that its loss leads to drug resistance) (33). Also, transporters are necessary for ion regulation (250).

SECRETORY PATHWAY AND SURFACE MEMBRANE PROTEIN BIOSYNTHESIS

Similarities with Other Eucaryotes: Transport of VSG

Synthesis of kinetoplast membrane and secreted proteins follows approximately the normal pathway for eucaryotic cells. Proteins synthesized on the rough endoplasmic reticulum pass to a Golgi apparatus and then to the flagellar pocket. Membrane proteins bear signal sequences which are cleaved upon import and can be predicted readily by standard algorithms, and mutational analysis of a signal peptide in *L. major* indicated that the functional requirements for such a sequence are indistinguishable from those found in other eucaryotes (216). Both N- and O-glycosylation pathways could be demonstrated in reactions with synthetic peptides as substrates (51) and have been seen on mature proteins, whose N glycosylation can be inhibited by tunicamycin (8, 67).

As usual, studies of *T. brucei* predominate, with particular attention having been paid to the biosynthesis of VSG. The biosynthesis of GPI-anchored macromolecules has been extensively reviewed elsewhere (61, 129). Acceptor proteins are synthesized with a hydrophobic C-terminal tail, which is replaced upon anchor addition. The anchors are synthesized and transferred to acceptors in the endoplasmic reticulum, where N glycosylation also occurs. VSG carbohydrate modifications occur sequentially in an intermediate compartment (9)—presumably the Golgi apparatus, which contains the expected glycosyltransferase activities (83). Transport of VSG was delayed but not abolished by reduced temperature (8), resulting in accumulation of VSG in a compartment assumed to be the *trans*-Golgi (57); monensin treatment caused swelling of these cisternae (57) and prevented full maturation of VSG (8). In higher eucaryotic cells, GPI-anchored proteins appear to be gathered together and transported in membrane microdomains or "rafts"; the GPI anchor plays a role in protein sorting in polarized cells (see reference 69 and references therein). Whether GPI anchors play any role in specialization of kinetoplastid surfaces is unknown, but it seems unlikely, because proteins with and without GPI anchors are found both on the external surface and on the flagellar pocket membrane. It is also not known whether the GPI-anchored and other surface or secreted proteins are secreted together or in separate, specialized vesicles.

Markers for the Secretory Pathway

A gene with homology to protein disulfide isomerase (a resident of the endoplasmic reticulum) and form I phosphoinositide-specific PLC has been cloned (98). The corresponding RNA is about 15-fold more abundant in bloodstream form trypanosomes than in procyclic forms, and the predicted protein has C-terminal −KQDL. This sequence is clearly related to consensus endoplasmic reticulum retention signals (49), but its function in trypanosomes has not been demonstrated and the location of the protein has not been confirmed. In contrast, retention of a *T. brucei* BiP/grp78 homolog in the endoplasmic reticulum has been demonstrated (10): the C-terminal tetrapeptide, −MDDL (also present on the *T. cruzi* homolog [215]), was essential for retention in the endoplasmic reticulum (7a), because its deletion resulted in secretion of the protein into the medium.

In *L. major* promastigotes, a protein bearing a 10-amino-acid repeat sequence is found in the endoplasmic reticulum and membraneous structures within the cytoplasm (227); this sequence is highly conserved among *Leishmania* species.

Export of the *L. major* Gene B Protein

The gene B protein of *L. major* is probably not exported to the cell surface by a conventional route: glycosylation was not detected, and no predicted signal sequence is present. Polyacrylamide gel electrophoresis revealed no strong difference in migration between the in vitro translation product and in vivo labelled gene B protein, although the two species were not run side by side on the same gel, so minor modifications cannot be ruled out. Both migrated anomalously slowly on polyacrylamide gels: the PARP of *T. brucei* behaved very similarly in this respect, and in neither case was the anomalous migration dependent on the presence of the repeat domain (70, 140). Despite the predicted hydrophilicity of the gene B sequence and the complete absence of a membrane-spanning domain or GPI attachment consensus sequence, the gene B protein partitioned into the hydrophobic phase upon Triton X-114 separation (70) and was associated with lipids during lipid fractionation. The fractionation behavior and membrane attachment are almost undoubtedly a consequence of a physical association between the gene B protein and LPG. Incubation of live, infective promastigotes with antibodies to LPG at 37°C resulted in capping, and the gene B protein was found associated with the capped LPG whereas other membrane proteins (such as gp63) remained distributed all over the surface. The gene B protein was clearly accessible to antibodies, whereas gp63 was efficiently masked from antibody access by the LPG being revealed only after the LPG had been capped (162). The homology between the gene B protein and the peptidoglycan-binding portion of *S. aureus* protein A (see above), together with the overall hydrophilicity, suggests that the gene B protein interacts with the glycan portion of LPG. What is still unclear is when the association occurs. One possibility is that the protein associates with LPG as it is excreted into the flagellar pocket; alternatively, the association may be a prerequisite for entry into the vesicular transport pathway. Glycolipid anchor precursors are synthesized on the cytoplasmic face of the endoplasmic reticulum (225) and must flip across the bilayer before transfer to proteins or addition of extended carbohydrate chains. (Thus, expression of a cytoplasmic GPI-PLC in *L. major* results in secretion of gp63 into the culture medium and a decrease in the amount of GPI precursors, although LPG synthesis appears unaffected [134].) If the gene B protein is associated at this early stage, it would have to be carried with the GPI precursor and flip with it. This seems unlikely: the accessibility of the protein to antibodies implies that it is associated with the glycan portion of the LPG and not with the anchor. Also, it is not associated with GPI-anchored gp63. Another question is what happens in amastigotes, where the LPG is very strongly down-regulated. Does the protein associate with glycoinositol-phospholipids or some other component such as high-molecular-weight phosphoglycans (240)? (*L. major* is the only species so far shown to have detectable LPG at the amastigote stage [240]. Is it the only one to have gene B?) Gene B protein is found in the flagellar pocket of amastigotes but not promastigotes; perhaps that is because there is not enough LPG to bind it all in amastigotes.

Secretion of proteins lacking signal sequences is not unprecedented, as it has also been seen for some mammalian proteins: in one example, a muscle lectin was exported in little membrane-bound blebs extruded from the cell surface (38).

Secretions

A variety of materials is found in flagellar pockets, but very few genuine secretions have been analyzed in any detail. This may be because there really are very few or because the aspect

has received little attention: one pitfall that may have discouraged such studies is the difficulty in distinguishing genuine secretions from molecules released by lysed, dead, or dying organisms. In the bloodstream stage of African trypanosomes, the pocket is filled with electron-dense carbohydrate-containing material (20) and acid phosphatase is cytochemically detectable (110); the pocket of procyclic forms appears much emptier, lacks acid phosphatase, and appears to be far less active in endo- and pinocytosis (110). Strings of acid phosphatase and high-molecular-weight proteophosphoglycan, as well as a fibrous network of complex phosphoglycans, can be seen streaming out of the flagellar pocket of *L. mexicana* (201). As mentioned above, *T. cruzi* secretes a hemolysin; maximal activity is found from amastigotes, but epimastigotes and trypomastigotes also make significant amounts (5). *Phytomonas* species secrete enzymes that degrade plant polysaccharides into in vitro culture medium; large (secretory?) vesicles are clustered round the base of the flagellar pocket (179).

FLAGELLAR POCKET AND RECEPTOR-MEDIATED ENDOCYTOSIS

General Characteristics of Endocytosis

Early studies in which ferritin was used as a tracer showed that it was taken up via the flagellar pocket and ended up in lysosomes (199). In *T. brucei* bloodstream forms, ferritin could be observed in budding coated vesicles, in a tubulovesicular endosomal network, and in the lysosomal compartment (110). Kinetic analyses by the Opperdoes laboratory with bloodstream form *T. brucei* and proteins adsorbed onto colloidal gold clearly distinguished passive "bulk phase" uptake of such molecules as ferritin or bovine serum albumin (BSA) from that of low-density lipoprotein (LDL), high-density lipoprotein (HDL), and transferrin (Tf) but not apo-Tf (44). The clearance rate for LDL was at least 2 orders of magnitude higher than that for BSA or ferritin, the HDL clearance rate was a little lower than that of LDL, and the Tf clearance rate was 10 times higher than that of BSA (44). These values suggest involvement of specific receptors for lipoproteins and Tf, an interpretation that was supported by the observation that binding of radiolabelled LDL and Tf was saturable at 4°C and could be specifically inhibited by excess unlabelled ligand. The putative LDL receptors appeared to be on the outer surface of the flagellar pocket membrane (44) and had a K_d of 5.7 nM (42), whereas Tf-Au was also very clearly in the flagellar pocket matrix.

In contrast to the observations described above, Vandeweerd and Black (221) found that bloodstream trypanosomes could take up tritiated phospholipids and cholesteryl esters from either HDL or LDL; label from radioiodinated apolipoproteins was not incorporated, although these clearly played a role in delivery of the tritiated components to the trypanosomes. The authors suggested that the lipids and cholesterol dissociated from the HDL or LDL within the flagellar pocket, perhaps on the trypanosome membrane (222). The discrepancy between these results and those of the Opperdoes laboratory may arise from the fact that the Opperdoes group starved the trypanosomes for HDL and LDL before doing uptake studies whereas the Black group did not; also, the Opperdoes group studies were done in the presence of BSA, which was reported by the Black group to inhibit lipid uptake (222). In contrast to other reports, Webster (230) could detect neither horseradish peroxidase nor Tf-Au in the flagellar pocket after a 30-min incubation at 0°C; this could be a consequence of different incubation times, because binding in the

cold is quite slow (44). After incubation at higher temperatures, the two tracers were clearly distributed to different subpopulations of vesicles and tubules. Some VSG-containing structures contained neither endocytosed horseradish peroxidase nor Tf-Au (231); these could be exocytic structures, which do not overlap with the endocytic pathway, or they could be vesicles specialized for VSG recycling. Metacyclic *T. congolense* behaved similarly to bloodstream forms; procyclic forms of both *T. congolense* and *T. brucei* could not endocytose Tf-Au or BSA-Au, although horseradish peroxidase was still internalized (231).

Subsequent studies involving immunoelectron microscopy, together with kinetic analyses, have yielded a more detailed picture of the endosomal network. As usual, different incubation temperatures could be used to distinguish endocytosis of BSA-Au into early endosomes (collecting tubules or tubular-vesicle compartment; inhibited at 4°C but still active at 12°C) from movement into the late endosomal/lysosomal compartment (inhibited at 12°C); the 12°C inhibition was accompanied by a change in morphology of the early compartment to give structures that were more rounded than before (24).

Coated vesicles are not seen in insect-living forms. Vesicles and coated pits that are morphologically very similar to those of mammalian cells have been observed budding from the flagellar pocket in bloodstream form trypanosomes (44, 110), but it is not at all clear just what they are coated with or whether they are equivalent to clathrin-coated vesicles. The trypanosome vesicles were larger than conventional coated vesicles (100 to 150 nm in diameter) and had an inner coat of electron-dense material which was probably VSG (187). By modification of a protocol for mammalian coated vesicles, Shapiro and Webster (187) were able to isolate a fraction highly enriched in the apparent *T. brucei* equivalent. Although the fraction contained a large number of different proteins, no band with the mobility of clathrin was seen. In an attempt to isolate a reliable marker, Webster and Shapiro (234) purified a bloodstream-trypanosome-specific, 77-kDa amphipathic protein by Triton X-114 extraction of a preparation of coated vesicle membranes. Unfortunately, this species was by no means restricted to coated vesicles, being located on the luminal face of lysosomal and many endosomal membranes.

LDL Receptors

The specific receptors for LDL and Tf in bloodstream-form *T. brucei* have been the subject of detailed investigation, largely because it was hoped that these unique molecules might represent targets for immunological or chemotherapeutic attack. The existence of a receptor for epidermal growth factor has been postulated, but much less is known (96). The LDL receptor purified from bloodstream-form *T. brucei* is a glycoprotein with a molecular mass in denaturing sodium dodecyl sulfate (SDS)-gels of 155 kDa; both purification of the cells on a DEAE column prior to isolation and omission of protease inhibitors resulted in production of a proteolytic fragment of 86 kDa (40) that retains the ability to bind LDL (42). Antibodies to the 86-kDa fragment slightly inhibited bloodstream trypanosome growth in vitro, the inhibition being reversible by high levels of LDL; and depletion of LDL from the medium increased the trypanosome doubling time. However, the doubling time of control parasites was significantly shorter than that seen in vivo or under more ideal culture conditions (42). The antibodies were able to inhibit the binding of LDL not only to various antigenic variants of bloodstream-form *T. brucei* but also to rat hepatocytes (41). The antiserum clearly strongly cross-reacted with native mammalian LDL receptor

but did not recognize the denatured mammalian protein on immunoblots (41). There are three possible evolutionary interpretations of this cross-reactivity: trypanosomes acquired the gene from the host; the common epitopes are a consequence of convergent evolution at the LDL-binding site; or (the least likely) the receptor arose very early in evolution and the ligand-binding region has been strongly conserved. Of eight monoclonal antibodies recognizing the 145-kDa trypanosome receptor, only one showed no significant binding to the rat LDL receptor (11). This antibody could be taken up by trypanosomes, and the uptake was competitively inhibited by LDL, suggesting that the antibody was entering via binding to the LDL receptor; uptake by rat fibroblasts was lower and nonspecific (11). This antibody (as well as a pool of monoclonal antibodies) was able to cause some complement-mediated lysis of trypanosomes, confirming that flagellar pocket antigens are accessible to the humoral immune system. Whether this accessibility has any role to play either in the real disease or in immunoprophylaxis, however, is questionable. Animals are clearly exposed to flagellar pocket antigens every time trypanosomes are lysed, as antibodies are made to successive VSGs, but they do not become immune.

Antibodies to the LDL receptor detected a protein in cultured procyclic forms with similar gel mobility to that of bloodstream forms. This is somewhat surprising, because there is no cholesterol in the tsetse fly fluids and procyclic forms are reported to be able to synthesize their own sterols (52). Lee et al. (113) cloned a *T. brucei* gene that encodes a protein (dubbed CRAM [cysteine-rich acidic integral membrane protein]) containing about 66 copies of a cysteine-and-aspartate-rich 12-amino-acid repeat. The repeat sequence shows significant similarity to mammalian LDL receptor sequences, and the protein was found by immunoelectron microscopy to be located on the flagellar pocket membrane—particularly, clustered around the base of the flagellum—and in vesicles just beneath. Because the mRNA and protein were expressed predominantly in procyclic forms, it was suggested that CRAM might be a procyclic LDL receptor. However, the molecular mass was much higher than that reported by Coppens et al. (40): a broad smear around 200 kDa rather than 145 kDa. (In bloodstream forms, only a very faint 100-kDa band was observed.) The size predicted from the sequence was 130 kDa, suggesting either in vivo glycosylation or abnormal migration of the protein in polyacrylamide gels or both. CRAM sequences were found in *T. cruzi* and in *T. equiperdum*, but the degree to which they are conserved or functional has not been determined; *T. equiperdum* has lost insect transmission ability, so it is not clear if CRAM is expressed or required by this species. The relationship between CRAM and the bloodstream form receptor will become clearer once the gene encoding the bloodstream form receptor has been cloned, and reverse genetic approaches will be needed to determine the role of each in the trypansome life cycle.

Transferrin Receptors

By far the best-characterized system for receptor-mediated endocytosis in kinetoplastids is that for Tf uptake. The major *T. brucei* Tf receptor is encoded by two expression site-associated genes, ESAG 6 and ESAG 7, which are not expressed in procyclic forms because of transcriptional regulation or attenuation (176). Affinity chromatography experiments, combined with N-terminal sequencing, initially implicated either ESAG 6 or ESAG 7 in Tf binding (182, 183). The two genes are closely related, being almost identical over the first 200 amino acids, and are conserved in different expression sites (200). The C

termini are less highly conserved, and ESAG 6 has a hydrophobic C-terminal extension which is absent from ESAG 7 (158) and serves as a signal for addition of a GPI anchor. It is now apparent that both gene products are required for Tf binding. Affinity-purified receptor or a complex precipitated by antibodies specific for ESAG 6 contained two polypeptides of 50 to 60 and 46 kDa (116, 178, 200), whose sizes decreased to 46 and 36 kDa, respectively, upon deglycosylation (200). Antibodies against the C terminus of ESAG 6 reacted with the larger species, whereas antibodies to the common N terminus reacted with both (200). Various lines of evidence confirmed that ESAG 6 has a GPI anchor. Affinity-purified ESAG 6 reacted with antibodies to the cross-reacting determinant (200). This epitope is exposed on GPI anchors that have been cleaved by PLC enzymes, such as the bloodstream form trypanosome GPI-PLC. After purification under conditions in which the endogenous GPI-PLC was inhibited, the intact ESAG 6/7 complex partitioned to the detergent phase in Triton X-114 separations but moved to the aqueous phase upon GPI-PLC treatment; ESAG 7 homodimers partitioned to the aqueous phase (200). Experiments in which the ESAG 6 and ESAG 7 genes were expressed in either procyclic trypanosomes (116), *Xenopus* oocytes (178), or insect cells (35) showed that both gene products were required for Tf binding, as measured by precipitation with Tf-Sepharose beads (116). In insect cells and microinjected oocytes, the complex was glycosylated, GPI anchored, and partitioned to the cell surface; the ESAG 6 product expressed alone also went to the cell surface, whereas expression of ESAG 7 alone yielded soluble cytoplasmic and excreted product (35, 178). Retention on the oocyte surface was mediated by the GPI anchor: transferral of the anchor to ESAG 7 induced plasma membrane localization and coexpression of ESAG 7 with an anchorless ESAG 6 yielded a receptor that was able to bind Tf but was secreted into the medium (178). It is clear that ESAG 6 and ESAG 7 can also homodimerize: by gel filtration, ESAG 7 alone or the soluble (GPI-anchorless) ESAG 6/7 complex, expressed in oocytes, had an apparent molecular mass of 80 kDa (dimer).

Interestingly, when ESAG 6 alone or the ESAG 6/7 complex was expressed in procyclic trypanosomes, it was distributed over the entire cell surface; clearly, the signal that mediates retention of the molecule in the flagellar pocket of bloodstream forms was not functioning (116). No substantial evidence for Tf endocytosis was observed in the procyclic double transfectant (116), consistent with earlier observations that these forms are in general not very endocytically active. (Perhaps coated vesicles are required for the endocytosis?)

When the ESAG 6/7 complex is expressed in its proper location—bloodstream trypanosomes—it does not behave as would be expected from all the above results. Antibodies to either Tf or the Tf receptor labelled predominantly the flagellar pocket matrix, with only minor surface labelling (116, 178, 200). That this was a fixation artifact is conceivable but unlikely: even if GPI-PLC remained active in the fixed state, it is in the wrong location for access to flagellar pocket proteins. When cell fractionation was performed in the presence of a GPI-PLC inhibitor, ESAG 6/7 complex was found in the soluble fraction, and its poor reactivity with anti-cross-reacting determinant antibodies suggested that the GPI anchor was retained (200).

The density of Tf receptor was estimated by Steverding et al. to be about 2,000 molecules per cell from the density of labelling in immunoelectron microscopy (200), in agreement with the estimates of 2,200 molecules per cell from Scatchard analysis and 3,300 molecules per cell from the yield on Tf Sepharose affinity chromatography (199a). These estimates are very

similar to the estimates, from Scatchard analysis, of 1,800 high-affinity LDL receptors per cell (42). Experiments involving radioactive Tf binding indicated that only one molecule of Tf bound per ESAG 6/7 dimer, under conditions where dimer dissociation was not expected (199a). In contrast, Salmon et al. (178) concluded from the results of gel filtration analyses that the anchorless ESAG 6/7 complex could bind either one or two molecules of Tf and estimated the number of receptors per cell, by Scatchard analysis, to be up to 27,000.

Despite the clarity of many of these results, some significant discrepancies remain. First, although Tf is a growth factor for bloodstream trypanosomes (181), it is not clear whether all bloodstream trypanosomes express this receptor all of the time: some flagellar pockets label with neither antibodies to ESAG 6 and ESAG 7 nor with Tf (200). Another problem is that metacyclic VSG expression sites completely lack functional ESAGs (1, 87). Expression of the metacyclic VSG persists for several days and many rounds of cell division once the trypanosomes are injected into the mammalian host. It is not known whether metacyclic forms have a Tf receptor at all. Possibilities are expression of ESAG 6/7 from some other expression site or from some other as yet undetected genomic site or expression of an alternative receptor. Grab et al. (84) isolated an apparently different 90-kDa protein by virtue of Tf binding. It was localized to the flagellar pocket and endosomes; in the presence of protease inhibitors, it was also in lysosomes, and a dimeric version (190 kDa) bound one molecule of diferric Tf. Other workers have not seen this species, and it is not clear what it is, but clearly the possibility that it is an alternative receptor (the ESAG 6/7 complex was clearly also present on the gels of affinity-purified receptor) cannot be ruled out. To determine this definitively, it will be necessary to shut off expression of the ESAG 6/7 complex in bloodstream trypanosomes (241).

Another interesting question is the role of the GPI anchor. Why is the ESAG 6/7 complex found preferentially in the flagellar pocket matrix and not on the membrane? How could it function in endocytosis? Does it merely serve to increase the level of Tf in the pocket, to facilitate fluid phase endocytosis, or does it jump back into the membrane? Or is only the minor membrane-bound component functional? How could a GPI-anchored species (spanning only the outer leaflet of the bilayer?) communicate with the cytoplasm to induce formation of coated pits and endocytosis? Normally, such communication occurs via a cytoplasmic portion of the receptor. These questions (several of which were raised in the above-mentioned publications) may to some degree be answered by reverse genetic approaches.

Fate of Transferrin and LDL Receptors

In eucaryotic cells, the Tf and LDL receptors are taken up upon ligand binding. Acidification of the endosomes results in dissociation of the ligand; the receptors then recycle to the plasma membrane for reuse. Many results up to now indicate that trypanosomes cannot do this: instead, everything is delivered to the lysosomes. The first indication that this was the case was the location of endocytosed Tf-Au. Experiments with gold particles of various sizes (down to Tf-Au5) yielded similar results in both *T. brucei* and *T. congolense* (230, 232), as did later immunolocalization studies with affinity-purified anti-bovine Tf immunoglobulin G. This rules out the possibility that the lysosomal localization was a consequence of misrouting of large gold particles. Native Tf, like Tf-Au, was found in the flagellar pocket, coated vesicles, tubular structures, and lysosome-like organelles (86); in one ultrastructural study, there

were indications that Tf-Au was sorted away from the VSG that had been simultaneously endocytosed (232). The concentration of Tf in *T. congolense* lysosomes could be confirmed by labelling with antibody to the lysosomal cysteine protease (86). Incubation of *T. brucei* with protease inhibitors resulted in a clear increase in the level of anti-Tf labelling, suggesting that normally the Tf is degraded by lysosomal proteases (86).

Coppens et al. (43) observed similar rapid degradation of trypanosome-associated ^{125}I-LDL; the degradation could be inhibited by leupeptin and E64, inhibitors of the trypanosomal lysosomal thiol protease (117), and also by chloroquine, which suggests that the digestion occurred in an acidic cell compartment (43). Chloroquine-mediated accumulation was inhibited by the ionophore monensin, which disrupts ion gradients, confirming that it was due to sequestration in an acidic compartment (43). Both monensin and chloroquine decreased the number of LDL receptors on the cell surface, and the effects were amplified in the presence of LDL; however, even at apparently saturating levels of monensin and LDL, 25% of LDL binding remained after 6 h, indicating either that the binding was mediated by receptors that could not be internalized or that some receptors were being recycled. Interestingly, procyclic trypanosomes could survive for several days in the presence of up to 25 μM chloroquine although the maximal cell density and division rate were decreased, with the 50% inhibitory concentration being 5 μM. Chloroquine inhibited bloodstream trypanosome growth in vivo.

Vandeweerd and Black (222) were, in contrast, able to detect neither trypanosome-associated HDL or LDL nor degradation products thereof; moreover, chloroquine and proteinase inhibitors had no effect, in their hands, on lipid uptake. However, there was a clear inhibition of cholesterol ester uptake upon inhibition of protein synthesis, which would be expected if continuous synthesis of new receptors is required.

HDL Uptake in African Trypanosomes

As noted above, *T. brucei* appears to have an HDL receptor as well as an LDL receptor, and both types of lipoprotein appear capable of delivering lipid to the cells. *T. brucei brucei* is killed by normal human serum, whereas the human pathogen *T. brucei rhodesiense* is not. The lytic factor is in specific subfractions of human HDL; what mediates its specificity is unknown. Hager et al. (88) have shown that *T. brucei brucei* takes up a trypanolytic factor into the lysosomes, whose membranes then appear to dissolve, suggesting that death occurs by autolysis. This factor has been purified; the active component is the human haptoglobin-related protein (189a). (The specificity of the purified lytic factor for *T. brucei brucei* was not demonstrated in those studies.) Significantly, the effects of the lytic component(s) could be inhibited by incubating the trypanosomes in weak bases or inhibitors of lysosomal proteases, clearly implicating low lysosomal pH and lysosomal proteolysis as critical factors in the toxic process (88, 120). Both *T. brucei brucei* and *T. brucei rhodesiense* appear to have high-affinity and low-affinity binding sites for HDL and are able to internalize it and concentrate it in intracellular vacuoles (74, 119). The resistance of *T. brucei rhodesiense* to the toxic effects of the factor presumably, then, lies downstream of internalization. Gillett and Owen (74) estimated that there were only 660 high-affinity binding sites for LDL per trypanosome in *T. brucei*, as opposed to 64,000 for human HDL and 11,500 for bovine HDL. These numbers are paradoxical in view of the clearance measurements of Coppens et al. (44), which implied that LDL clearance is faster than that of HDL. One possible explanation could be that not all binding sites are involved in

internalization. Binding of HDL to *T. cruzi* trypomastigotes via surface neuraminidase has also been reported (168); epimastigotes bound somewhat less, and proteolysis did not remove the bound label, perhaps indicative of internalization.

Receptors as Vaccines?

The work on both the Tf and LDL receptors was initiated partially with a view to possible immunization. If these receptors are required, they must be relatively invariable, as the binding and internalization functions must be conserved. This would make them attractive vaccine candidates. In this context, the coupling of ESAG 6/7 to VSG expression is interesting, because there is presumably at least one copy of ESAG 6 and ESAG 7 per bloodstream expression site. Comparison of the three ESAG 6 sequences available reveals a very small (32-nucleotide) sequence that is highly variable. If this sequence were exposed on the receptor surface (it is presumably not required for receptor function), it might partially serve as an additional form of antigenic variation, enabling evasion of the immune response (19). However, ESAG 7 does not have this variability, and it seems unlikely that variation of a very small peptide loop could divert attention from the rest of the molecule, especially in the flagellar pocket matrix. Is it possible that the diversion of all receptors to the lysosome and the absence of recycling constitute a mechanism that protects the trypanosomes against the immune response? That way, receptors become dispensable components that can be either shed or rapidly destroyed if necessary—all the parasite has to do is overproduce them.

Endocytic Pathway in Kinetoplastids Other than *T. brucei*

We have found no detailed reports on receptor-mediated endocytosis in other kinetoplastids. *L. mexicana* amastigotes are able to endocytose material from the lumen of the host cell lysosome (177). *T. cruzi* epimastigotes were able to endocytose horseradish peroxidase (50), and also Tf-Au (190)—at a guess, this occurs by nonspecific bulk fluid phase endocytosis, because epimastigotes live in the insect gut, although use of Tf from ingested blood cannot be ruled out. The gold label was found in the flagellar pocket and at the base of the cytosome, and clathrin-coated vesicles were absent.

PROTEINS OF THE LYSOSOMES AND THE ENDOSOMAL NETWORK

As already noted, in most trypanosomatid species there are not yet enough markers to enable different vesicular compartments to be identified with certainty. A number of macromolecules have been reported to be localized in the endosomal network; most of them are molecules in search of a function whose sequences are not yet known. The relationships between the different markers (if any) have yet to be elucidated. Lysosomes have been identified by virtue of enzymatic markers such as acid phosphatase and other hydrolases (198) and also as the final destination of endocytosed material such as ferritin, horseradish peroxidase, and BSA-Au (110, 138, 230). Even these endocytosed materials did not always colocalize, suggesting further specialization within the endocytic network (127). The pH of *T. brucei rhodesiense* lysosomes was estimated (by fluorescence quenching) to be 6.0 to 6.1, almost 1 unit higher than that of mammalian lysosomes (24), but from chloroquine accumulation data, the pH of *T. brucei brucei* bloodstream form lysosomes was calculated to be about 4.5 (43).

It is not known how trypanosomatid lysosomal proteins are sorted to the correct destination, and the subject has received almost no attention. In mammalian cells, many lysosomal enzymes are modified by specific phosphorylation of mannose residues. The mannose 6-phosphate is recognized by mannose 6-phosphate receptors which mediate vesicular transport to the lysosomes. At this point, the enzymes and receptors can dissociate, allowing recycling of the receptors. A proportion of the lysosomal proteins escapes this selection and is excreted, but these proteins can be scavenged by surface mannose 6-phosphate receptors (reviewed in reference 93). This pathway may not exist in trypanosomatids—at least, not in a mannose 6-phosphate-dependent form. Cazzulo et al. were unable to detect mannose 6-phosphate in the *T. cruzi* protease cruzipain by various chromatographic and enzymic methods (34); in fact, they could detect no mannose 6-phosphate at all in this organism. However, several other lysosomal targeting mechanisms exist in higher eucaryotes, and sorting of proteins to the *Saccharomyces cerevisiae* vacuole does not depend on mannose 6-phosphate (97, 122). Mammalian lysosomal membrane proteins do not depend on mannose 6-phosphate for sorting (160); they can cycle to and from the cell surface, the return to lysosomes being determined by cytoplasmic domains that are recognized by adaptor proteins (160). Even nonglycosylated proteins can be directed to lysosomes—presumably directly from the cytoplasm (93).

Cysteine proteases have been shown to cofractionate with lysosomes of *T. brucei* (118), *T. congolense* (127), *T. cruzi* (58, 180), and *Leishmania* species (see reference 56 and references therein). In most cases, the localization has been confirmed by immunoelectron microscopy and/or colocalization with endocytosed molecules; usually, a proportion of the immunoreactive material was also in the flagellar pocket. The major lysosomal cysteine proteases share about 42% amino acid similarity in the catalytic domain and bear an unusual C-terminal extension (139) which is only 19% conserved between species. The major cysteine protease of *L. mexicana* amastigotes, encoded by multicopy lpcys 2 genes in *L. mexicana pifanoi* and LmCPb genes in *L. mexicana mexicana*, was found in large lysosomes called megasomes that are unique to these species and occupy as much as 15% of the cell volume (56); only a small proportion of the product was in vesicles and in the flagellar pocket. The lpcys 1 and LmCPa genes are less related to the genes of other species, are present in only one or two copies, and encode a less abundant protein, which was found partially in the megasomes and partially on the cell surface, particularly in the flagellar pocket. The LmCPa gene encodes an enzyme with only a short (9-amino-acid) C-terminal extension; mutants in which this gene had been deleted had no discernible growth phenotype either in vivo or in vitro (196). Release of leishmanial proteases, either by active secretion or after parasite lysis, may play a role in lesion pathology (99).

The major *T. cruzi* epimastigote cysteine protease has been called cruzipain or cruzain (58); it, too, may have roles in infectivity and pathology (180). The protein is concentrated in reservosomes, large lysosome-like organelles toward the posterior of epimastigotes with an internal pH of 6.0. The organelles have been classified as prelysosomal, because they lack acid phosphatase (58); however, definition of the compartment was difficult because antibodies to mammalian lysosomal markers (LAMP 1, LAMP 2, and lgp120) did not cross-react with the cells at all.

Like many other lysosomal enzymes in higher eucaryotic cells (93), these proteases are multiply processed: after initial removal of the presequence, the proenzymes are transported toward the lysosomes where (auto?)proteolytic removal of an N-terminal segment occurs. In some species, a third proteolytic step within the lysosomes or megasomes removes the C-termi-

nal extension. This happens to the lpcys2 product, the extension of which is readily detectable as a separate entity in pulse-labelling experiments (56). It has been suggested that the C-terminal extension—which appears neither essential for, nor inhibitory of, enzyme activity—functions in organellar targeting, but there is as yet no evidence for this, and the sequence possesses no motif that is similar to the higher eucaryotic consensus for adaptor recognition.

Several other markers of unknown function and/or sequence have demonstrated lysosomal or endosomal association. The largest abundant membrane protein purified from *T. brucei* endocytic vesicles with Triton X-114 was a 77-kDa doublet, which was ultrastructurally localized to the membranes of endosomes and lysosomes (234). Indirect immunofluorescence with confocal image analysis and immunoelectron microscopy located *T. brucei* proteins bearing an EARLRAEE motif to a membranous network. These Tb-29 proteins were most abundantly distributed to the area surrounding the nucleus, the region between the nucleus and the flagellar pocket, and the region immediately underneath the flagellar pocket membrane (111). A monoclonal antibody raised to fixed procyclic forms of *T. vivax* reacted with a 65-kDa integral membrane glycoprotein present in all life cycle stages, bearing N- and O-linked glycans and associated primarily with membranes of vesicles that were part of the endocytic network, as they contained endocytosed BSA-Au but not significant amounts of VSG (30). The epitope was probably proteinaceous and was specific to *T. vivax*, being undetectable in other trypanosomatids; a 22-kDa molecule also bore the epitope, but its relationship with the 65-kDa molecule is unknown.

One marker for late endosomes in bloodstream form *T. brucei* was identified with a monoclonal antibody, CB1 (21). The corresponding protein was detected in pulse-chase experiments as a 57-kDa band which acquired N-linked oligosaccharides (bearing the CB1 epitope) 20 to 30 min after synthesis. By surface biotinylation, it was shown that a significant proportion of this product was exported to the flagellar pocket before being routed through the collecting tubules to the late endosomal or lysosomal compartment, where partial degradation/processing to a 42-kDa form was observed (22). This routing is clearly similar to the pathway taken by some mammalian lysosomal proteins. The CB1 epitope is more abundant in stumpy bloodstream forms than in long slender bloodstream forms and is absent from procyclic forms (23). Whether this reflects absence of the core protein or merely regulation of glycosylation is unknown. Similarly, the sequence and function of the protein are unknown.

The status of a 66-kDa *Leishmania* antigen—purified by virtue of glutathione binding—is a little more difficult to assess (246). Antibodies to this preparation reacted with a number of different protein bands on Western immunoblots or by immunoprecipitation; the antigens are detected by immunoelectron microscopy on the surface of *L. major* promastigotes and in the parasitophorous vacuole but most predominantly in tubulovesicular cisternae and the multivesicular megasomes. Unfortunately, affinity-purified antibodies against the original 66-kDa immunogen failed to react on *L. major* cryosections, although patchy binding to many intracellular structures of several trypanosomatid species was observed by immunofluorescence.

NUCLEUS

Nuclear import in kinetoplastids has not been specifically investigated. However, it is known that bacteriophage T3 and T7 polymerases and the *lac* repressor can be directed into the nucleus by the nuclear localization signal of simian virus 40

virus T antigen (14a, 242). It has also been reported that a *T. cruzi* Hsp70 is translocated to the nucleus upon heat shock (123).

MITOCHONDRION

Structure and Component Proteins

There is only one mitochondrion per cell; division of the kinetoplast DNA and of the basal body precedes nuclear division and the outgrowth of a new flagellum (188). In many trypanosomatids, the mitochondrion extends the whole length of the cell and has a complex branched structure; internally, there are lamellar or tubular cristae. This, combined with the robust structure of kinetoplastids, makes isolation of intact mitochondria almost impossible: any procedure vigorous enough to break the cells disrupts the mitochondria as well. However, it is possible to obtain resealed mitochondrial vesicles that retain at least some enzymatic activities (91, 149). Differential detergent permeabilization can also be used for some purposes (53). The mitochondrion of bloodstream salivarian trypanosomes is rudimentary, lacking most Krebs cycle enzymes and oxidative phosphorylation.

As in other eucaryotic cells, some mitochondrial proteins are encoded by the mitochondrial DNA and the rest are imported from the cytoplasm. Distinguishing which are imported is, however, more complicated for kinetoplastid protozoa than for other species. The mitochondrial DNA of kinetoplastids consists of a network of maxicircles (20 to 40 kb) and hundreds of minicircles (0.7 to 2.5 kb). The protein-coding regions of the kinetoplast DNA are all to be found on the maxicircles, but many of the open reading frames are incomplete. To complete the open reading frames, the RNAs must be posttranscriptionally edited, using as templates small "guide RNAs" transcribed from maxicircles and minicircles (203). Some transcripts are so extensively edited that the nature of the polypeptide encoded may be impossible to discern unless cDNAs are found; indeed, such cryptogenes are usually only recognizable by their unusual nucleotide composition. Homologs for mitochondrial genes from other species may therefore be impossible to distinguish on the kinetoplast genome. Nevertheless, many mitochondrially encoded proteins have now been identified; they are similar to those found in other species (see, for example, reference 204 and references therein). Relatively few nucleus-encoded mitochondrial proteins have been investigated in trypanosomatids; these are described below.

Import of Proteins into Mitochondria in Other Species

As the mitochondrion is enclosed by a double membrane, mitochondrial proteins can be subdivided into those destined for the outer membrane, inner membrane (and cristae), intermembrane space, and matrix. Import of proteins into the mitochondria occurs posttranslationally in vivo and in vitro (reviewed in references 90 and 105), although some studies of *S. cerevisiae* strongly suggest that at least some import is cotranslational (71). Import into the matrix requires ATP and a membrane potential. Proteins destined for the matrix bear signal sequences about 20 amino acids long, which can be recognized by virtue of their (potential) ability to form an amphipathic helix (175). The polypeptide is imported in an extended conformation across specialized contact sites between the inner and outer membrane; upon import, the signal sequence is cleaved by a matrix protease (the MAS protease in *S. cerevisiae*). At least some polypeptides become associated with mitochondrial chaperones, which are involved in folding the pro-

teins in the correct conformation and ensuring unfolding and detachment from cytoplasmic chaperones (205). There are several pathways for directing proteins to the mitochondrial membranes and the intermembrane space (76, 77, 155, 237). Outer membrane proteins lack matrix-targeting signals and are inserted directly in a process that may or may not involve components of the general import machinery (depending on the protein). Of proteins destined for the intermembrane space, some, such as the iron-sulfur protein of the cytochrome bc_1 complex, have a matrix-targeting sequence and are imported all the way into the matrix, with signal sequence removal, before reinsertion into the inner membrane. During the transfer of the protein to the inner membrane, a second cleavage removes another 8 or 9 residues from the N terminus. Other proteins of the intermembrane space, such as cytochrome c and adenylate kinase, have no cleaved presequence; cytochrome c crosses the membrane directly without using surface receptor proteins or ATP. Considerable controversy has surrounded the import of a third class of proteins, epitomized by cytochromes b_2 and c_1. It is clear that cytochrome b_2 is inserted far enough across both membranes for cleavage of the signal sequence by the matrix protease and that neither matrix ATP nor the mitochondrial chaperone mhsp60 is required for the proteins to reach their final destination (226). The controversy centres around the question whether the proteins pass through the matrix and are rapidly reexported into the intermembrane space (the reexport model) or whether they are released from the channel without ever entering the matrix (the stop-transfer model) (77, 237).

Mitochondrial Import in Kinetoplastids

Our data indicate that many features of the mitochondrial matrix import apparatus are conserved in *T. brucei*: a hybrid protein consisting of the yeast cytochrome oxidase subunit IV presequence fused to dihydrofolate reductase is efficiently imported into procyclic trypanosome mitochondria, and the signal sequence is cleaved (94). Nuclear genes encoding a number of mitochondrial matrix proteins have been cloned from kinetoplastids. Often, although not always, the presence of a signal sequence could be predicted, but the cleavage of the sequence has been confirmed in only a few instances and none has been functionally tested. The putative signal peptides that are absent from the mature proteins are sometimes exceptionally short—only 8 or 9 amino acids.

Kinetoplastid Chaperones

Chaperones are important in ensuring correct folding of soluble proteins and can also play a role in maintaining proteins that are destined for transport across membranes in a transport-competent conformation. Various putative chaperones (or their genes) have been identified in kinetoplastids; their role(s), if any, in protein folding and import of proteins into organelles has yet to be defined. For example, hsp70 homologs have been cloned from *T. cruzi* (147), *T. brucei* (75), *L. amazonensis* (17), and *L. major* (112, 186). The *T. cruzi* hsp70 has very high ATPase activity, consistent with a chaperone-like function (147). In *L. major*, newly synthesized gene B protein coimmunoprecipitated with several proteins in the 70-kDa size range, at least some of which comigrate with hsp70-related polypeptides (70). Several mitochondrial chaperones have also emerged from homology searches: mhsp70 homologs from *C. fasciculata* (59), *T. cruzi* (62, 63, 147), *L. major* (185), and *T. brucei* (10). The putative *C. fasciculata* mhsp70 bears a 20-amino-acid sequence that is absent in the mature protein; this mhsp70 is concentrated around the kinetoplast DNA and

has 84% homology to yeast mtp70 and 56% homology to *Escherichia coli* DnaK. A putative 23-amino-acid targeting signal is also found on the *L. major* mhsp70, but this sequence probably could not form an amphipathic helix (185). The *T. cruzi* mtp70, a 71-kDa protein with a predicted signal of 25 amino acids, localized to the periphery of the kinetoplast, not the whole organelle (63). The authors therefore suggested, on the basis of a 55% homology to the DnaK protein of *E. coli*, that this protein might have some role in kinetoplast DNA replication. The homology to DnaK extends to function inasmuch as both proteins can autophosphorylate in the presence of calcium ions (147). In contrast, the *L. major* mhsp70 has predicted ATP-binding and calmodulin-binding domains, as expected for such a protein, and has been immunologically localized to the whole mitochondrion and kinetoplast DNA region. In addition, the sequence of a putative *T. cruzi* mhsp60 has been reported (73); this protein has 41% homology to GroEL and 52% to yeast hsp60. Using the GroEL homology, the predicted signal sequence is only 9 amino acids.

Proteins Associated with the Kinetoplast

The presence in trypanosomes of a complex kinetoplast network ensures that the subset of mitochondrial proteins required for network replication is concentrated within or near the kinetoplast DNA. *C. fasciculata* contains a topoisomerase II, active as a homodimer in ATP-dependent decatenation of minicircles from kinetoplast DNA networks (157) and associated with kinetoplast DNA in vivo (133). The predicted protein of 138 kDa has 68% homology to *T. brucei* topoisomerase II (202); hybridization revealed a single nuclear gene in both organisms, but homologs with limited sequence similarity would not have been detected. No signal peptide is obvious from the predicted amino acid sequence.

Another kinetoplast-associated protein is the KAP of *T. cruzi* (80). The predicted protein was expressed in epimastigotes and amastigotes but not circulating trypomastigotes. A molecular mass of 118 kDa was estimated from the sequence. Antibodies to an N-terminal peptide reacted on immunoblots with a 75-kDa polypeptide and recognized a protein inside the kinetoplast. However, this N-terminal sequence resembles a mitochondrial signal sequence, which one would expect to be absent from the mature protein. There are some discrepancies here that merit further investigation.

Additional proteins have been isolated from *C. fasciculata* by in vivo formaldehyde cross-linking followed by kDNA isolation. From the isolation methodology, these proteins are presumably associated with the kinetoplast. Five species of 15, 16, 17, 18, and 21 kDa were distinguished; by comparison of the cDNA and N-terminal peptide sequences of p16, p17, and p18, it was clear that 8 or 9 amino acids had been removed (245). This—like the peptide cleaved from *T. cruzi* mhsp60—is very short for a signal sequence; nevertheless, the peptides do have features reminiscent of signals, i.e., a hydrophobic residue preceding the cleavage site and Ser/Thr at position −5. p16 and p17 are very basic and were suggested to function in compaction of kDNA. So far, no information is available concerning the mechanism of specific sublocalization of proteins to the kinetoplast region of the mitochondrion.

Cytochromes and Other Proteins

Normally, cytochrome c reductase consists of nine bands, one of which is cytochrome b and another of which is cytochrome c_1. The *C. fasciculata* complex also has nine subunits; although their amino-terminal sequences did not include that of cytochrome b (165), it was clearly there by spectroscopy.

Partial or complete clones of the cytochrome c_1 gene have been isolated from a number of trypanosomatids; the sequences predict proteins that would be able to form only one thioether bond with heme, instead of the normal two (165). The cytochromes c_1 from *C. fasciculata* and *Bodo caudatus* lack cleaved signal sequences, in contrast with the yeast and human enzymes but similar to the genus *Euglena*, which is evolutionarily relatively near the kinetoplastids (167); in both *Euglena* and *Crithidia* species, only the initiator methionine is removed (167). Two more subunits of cytochrome *c* reductase were identified from *C. fasciculata*, with gel mobilities of 51 and 38 kDa (165, 166). In addition, a *T. brucei* homolog of the NdhK subunit of NADH dehydrogenase has been cloned; it is a nuclear gene, and both the function and the putative 9-amino-acid signal sequence (which could form an amphipathic helix) are based only on predictions from the translated gene sequence (161). Although this gene is found in the mitochondrial DNAs of some species, a nuclear location would not be without precedent, as the bovine gene is also nuclear.

In *T. brucei*, cytochrome *c* mRNA is present in both bloodstream and procyclic forms (218), showing about fivefold up-regulation in procyclics, but the activity is much more strongly developmentally regulated: the protein is extremely unstable in bloodstream forms (217). It is in fact not clear whether the protein is imported into mitochondria at all in bloodstream forms or is degraded as soon as it is made in the cytoplasm—raising the intriguing possibility that import (or lack of it) contributes to the regulation. Similar considerations appear to obtain for cytochrome *c* reductase subunits 2 and 4 and NdhK. During differentiation of stumpy forms to procyclics, the protein appears about 6 h after initiation of the differentiation; cytochrome *b*, in contrast, is already present in stumpy forms (166).

It is not clear how well the mitochondria of bloodstream form trypanosomes function in import. A membrane potential is clearly present, although it seems likely to be weaker than that seen in procyclics: bloodstream mitochondria are able to concentrate rhodamine present at low levels, whereas procyclic mitochondria can absorb much more from the cytoplasm (reviewed in reference 213). Assays of the F_1F_0 ATPase indicate that a membrane potential is maintained in bloodstream forms (145, 239); in fact, the mere presence of this activity indicates that import is functional, because only one subunit of the ATPase is encoded by the maxicircle. We have found that the dihydrofolate reductase fusion protein bearing a yeast cytochrome oxidase subunit IV signal does become associated with a pellet fraction when expressed at low levels in bloodstream forms and appears to be cleaved to the mature form, but immunolocalization has so far proved difficult because of low expression (93a).

GLYCOSOME

General Features

The glycosomes of kinetoplastids are evolutionarily related to the peroxisomes and glyoxysomes of yeasts, multicellular animals, and plants; together, these organelles are termed microbodies. They are spherical or elongated organelles bounded by a single unit membrane and sediment at a density of about 1.23 g/ml in sucrose density gradients (18, 148). The size and enzymatic content vary according to species and life cycle stage. The contents include classical peroxisome components, such as enzymes of fatty acid oxidation (ether lipid biosynthesis) and pyrimidine synthesis; malate dehydrogenase and ad-

enylate kinase may also be associated with the organelle (150). Catalase is absent from the parasites of mammals but present in *Crithidia* (142, 149) and *Phytomonas* (179) species. The most remarkable feature of glycosomes is that they contain the first seven enzymes of glycolysis and two of glycerol metabolism (151, 179; reviewed in reference 65). As in yeast and mammalian peroxisomes, the enzymes of the glycosome may form cores of apparently ordered structure that are stable even after organelle permeabilization (137).

Not only the morphology but also the biogenesis of microbodies has been conserved throughout evolution. Proteins are synthesized on cytoplasmic polyribosomes and imported posttranslationally into the organelles without any obligatory post-translational modification (18, 206). Two types of peroxisomal signal sequences have been recognized (47). One, the PTS-1, consists of the C-terminal sequence −SKL and related peptides (81, 82); another, the PTS-2, was first identified at the N terminus of rat 3-ketoacyl coenzyme A thiolase (209) but has since been identified on other enzymes. The consensus so far for this signal, starting no more than 10 residues from the initiator methionine, is charged-R/K-small hydrophobic-x-x-small hydrophobic-x-x-H/Q-L; mutagenesis shows that the three most highly conserved residues are essential for efficient import (78). One peroxisomal component has both types of signal (229); others may have a third, internal, or bipartite type of signal (107, 189). Results with yeast mutants (108, 219) and permeabilized mammalian cells (235) suggest a model—still very speculative—in which the PTS-1 and PTS-2 signals may be recognized by cytoplasmic receptors that deliver the signal-bearing proteins to the import machinery on the peroxisomal membrane (124, 128). The fact that many peroxisomal proteins bear C-terminal signals precludes cotranslational import and implies either that they are delivered to the import machinery in a folded state or that folding in the cytoplasm is actively prevented—for example, by cytoplasmic chaperones (228).

Glycosomal Import Signals

Most studies of the glycosomal import process have been done with *T. brucei*, because of the relative ease with which bloodstream trypanosomes can be broken and the organelles can be isolated. Import was shown to be posttranslational and to occur without substrate be without modification within a few minutes of synthesis (36, 92). The nature of import signals was originally deduced from the sequences of glycosomal enzyme genes, very many of which have now been cloned from several species (reviewed in reference 195). Initial comparisons between trypanosome enzymes and their cytoplasmic counterparts from other species suggested a model in which the signal was composed of two hot spots of basic residues 40 Å (4 nm) apart (238). Although this model subsequently turned out to be incorrect, it was prescient as it implied that the proteins were recognized in a mature folded state—something that now seems almost certain to be correct, at least for proteins bearing C-terminal PTS-1 signals. Perversely, this was the main reason why the hypothesis was greeted with considerable skepticism at the time! The reason for the presence of the hot spots and for the overall larger size and excess positive charge of glycosomal enzymes is still a mystery.

T. brucei and many other trypanosomatids have three genes encoding isozymes of phosphoglycerate kinase (PGK). The first, the A gene, is present in glycosomes in low relative abundance (2, 210); preliminary data suggest that its signal sequence is somewhere within the N-terminal 91 amino acids (156). The B gene encodes a cytoplasmic enzyme, and the C

TABLE 1. Sequenced enzymes of kinetoplastids that are (sometimes) glycosomal

Enzyme	Organism	Charge[a]	Signal	Comments	Reference(s)
Phosphoenolpyruvate carboxykinase	*T. brucei*	+9.89	−SRL[b]	Second signal also present; deletion of −SRL does not prevent import	192
	T. cruzi	+8.27	−ARL[c]		
Glyceraldehyde phosphate dehydrogenase	*T. brucei*	+13.22	−AKL[b]	A cytoplasmic enzyme is only distantly related	135
	L. mexicana	+9.22	−SKM[c]		89
	T. cruzi	+7.46	−ARL[c]		104
	T. borelli	+10.42	−AKL[c]	Two isoenzymes differ in 17 residues; both end in −AKL; 30% in glycosomes, 70% in cytosol	236
		+8.42			
Phosphoglucose isomerase	*T. brucei*	+2.48	−SHL[b]	Glycosomal in bloodstream forms; in procyclics, a considerable portion is in the cytosol	121
	L. mexicana	−3.58	−AHL[b]	More than 90% cytosolic	
	C. lucilae	NA[d]		All in cytosol	146
PGK-C	*T. brucei*	+14.66	−NRWSSL[b]	−SSL alone weak; R → E abolishes import	16, 193
	C. fasciculata	+1.81	MVLASP[e]	Putative signal nonfunctional in *T. brucei*	207
	L. major	+12.05	STKLIR[e]	No C-terminal homology to PTS-1	GenBank L25121
Fructose bisphosphate aldolase	*T. brucei*	+11.52	N-terminal PTS-2[b]		15
	L. mexicana	NA	N-terminal PTS-2[f]		134a
PGK-A	*T. brucei*	+9.89	Unknown		2
	C. fasciculata	−0.12	Unknown	Location unknown	210
Triose-phosphate isomerase	*T. brucei*	+6.06	Unknown	Glycosomal	208
	L. mexicana	+3.25	Unknown	Cytoplasmic and glycosomal	106

[a] Calculated with the Genetics Computer Group Sequence Analysis Software Package.
[b] Proven function in the homologous organism.
[c] Assumed based on homology to PTS-1 signals in T. brucei.
[d] NA, not available.
[e] Deduced from comparison with cytoplasmic isozyme.
[f] Deduced from PTS-2 homology.

gene encodes its glycosomal counterpart. The most obvious difference between B and C genes is the presence in the C gene of a C-terminal extension (153). Classical gene fusion experiments, in which this sequence was attached to the C terminus of chloramphenicol acetyltransferase and the fusion protein was expressed in *T. brucei*, demonstrated that the last 22 amino acids of PGK-C were sufficient to direct import of the protein into glycosomes (72). Subsequent mutagenic analyses, with variants of PTS-1 signals joined to luciferase, β-glucuronidase, or chloramphenicol acetyltransferase, showed that *T. brucei* recognizes peroxisomal entry signals. The spectrum of amino acid changes that is permitted is rather broader than that allowed in mammalian cells, but the amino acids preceding the C-terminal tripeptide can also influence the efficiency of glycosomal import (16, 191, 193). Recently, we have shown that *T. brucei* uses the PTS-2 pathway as well: the signal sequence of rat 3-ketoacyl coenzyme A thiolase is functional, and *T. brucei* fructose bisphosphate aldolase has a functional N-terminal PTS-2. As in *S. cerevisiae* (78), no N-terminal processing takes place (36). The essential residues of PTS-2 are conserved on the N terminus of *L. mexicana* aldolase (134a).

As for peroxisomes, the vast majority of glycosomal enzymes so far sequenced bear PTS-1 type signals (Table 1). Many different variants of the signal are present. Oddly, the consensus −SKL itself has not been found, although −SKL-specific antibodies clearly react with the organelle matrix (103). The long evolutionary history of the kinetoplastids has also allowed subtle variations between species in signal utilization. This can be seen from the list of kinetoplastid PTS-1 signals in Table 1. For example, *C. fasciculata* PGK-C has a 38-amino-acid C-terminal sequence that terminates in −ASP (207), a combination predicted not to function in *T. brucei* (193). Preliminary results from expression studies confirm this prediction (54), but this interpretation is weak without complementary experiments with *C. fasciculata*.

In *L. mexicana*, at least two enzymes have unexpected localization. The glucosephosphate isomerase has C-terminal −AHL, a PTS-1 signal that is fully functional in both *T. brucei* and other eucaryotes. However, nearly all the glucosephosphate isomerase in *L. mexicana* is cytoplasmic (146). Either *L. mexicana* has an inefficient import apparatus or this is another example of divergence in the efficiency with which different signal sequences are used. (There is no other sequence difference nearby that might account for the weak function.) Triose-phosphate isomerase, whose signal sequence is unknown, is also found in both compartments (106).

Import Mechanism

Detailed experiments on the mechanism of glycosomal protein import have made little progress despite considerable effort (195). Attempts to develop in vitro import methodology made little headway in several laboratories, being impeded by the difficulty of isolating intact organelles, the apparent "stickiness" of the glycosomal membrane, and the protease resistance of glycosomal proteins. Dovey et al. (55) did, however, succeed in demonstrating an association of in vitro synthesized PGK-C with isolated glycosomes. Up to 50% of added labelled PGK-C became resistant to extraction by 3 M urea and to digestion by proteinase K (500 μg/ml). The input protein became associated with SDS-resistant glycosomal protein cores and could be chemically cross-linked to endogenous glycosomal proteins (194). The association was specific—with PGK-B, it did not happen (55). Two observations suggested that the denatured (or less tightly folded) protein was a superior substrate for the glycosomal association. Prior denaturation of the PGK-C enhanced its apparent import, and native "cold" PGK-C could not compete with the in vitro substrate unless it was denatured prior to addition. Doubts remain because the PGK-C could also bind directly to membraneless glycosomal protein cores, albeit yielding a complex of much higher density than that obtained when intact glycosomes were used. Also, although the assay is time and temperature dependent, it apparently does not require ATP (194), whereas import into peroxisomes does (100, 235). All these studies were conducted before signal sequences for microbody import had been defined. It would be worthwhile to revisit the topic now that it is possible to build more protease-sensitive import substrates and to use alternative methods for selective cell permeabilization (235).

We have studied the unfolding requirements for glycosomal import with a dihydrofolate reductase fusion protein. Methotrexate binds with high affinity to this protein, inhibiting unfolding. Dihydrofolate reductase coupled to a mitochondrial targeting signal can be imported into mitochondria, but upon addition of methotrexate, the stably folded complex gets stuck in the import channel (60). Our results indicate that binding of the drug to dihydrofolate reductase in vivo does not inhibit import of the protein into glycosomes, implying that either the protein enters in the folded state or there is a strong unfolding activity associated with the glycosomal membrane (94). We have searched without success for trypanosome genes that have sequence similarity to *S. cerevisiae* genes involved in peroxisome biogenesis (14b).

Why the Glycosome?

The glycosome was originally discovered in bloodstream forms of salivarian trypanosomes. Here it seemed logical to package glycolysis in an organelle: intermediates would be locally trapped, resulting in a very high rate of glycolysis. This would be a good adaptation for an organism living in a bath of glucose and lacking oxidative phosphorylation. However, the glycosome has been conserved in all kinetoplastids examined, including monogenetic species, the members of the *Bodonidae* (151), and the plant-parasitic genus *Phytomonas* (179), suggesting that it was present in the progenitor of both parasitic and nonparasitic species (68). The origin of glycosomes, along with that of peroxisomes and glyoxysomes, is shrouded in mystery, and it is not at all clear what advantage the compartmentation of glycolysis confers on kinetoplastids. A particularly interesting case is the major glycosomal PGK isozyme, which is not required in, for example, procyclic *T. brucei*. It transpires that in at least one strain of *T. congolense*, a frameshift mutation has

resulted in premature termination of glycosomal PGK homolog translation before the C-terminal signal. As a consequence, the only glycosomal PGK in *T. congolense* is the "A" isozyme (156). Either very little or perhaps no glycosomal PGK is needed in these parasites. The transfer of other glycosomal enzymes to the cytoplasm will show which metabolites can cross the glycosomal membrane. Reverse genetic approaches should also reveal the role of glycosomal compartmentation, if genes required for glycosomal biogenesis can be isolated. The fact that glycosomal mutants of procyclic trypanosomes can be isolated (195) shows that the glycosome is not essential, at least in this form in vitro. Will glycosomeless bloodstream forms also survive? If so, why has the compartmentation been conserved?

SUMMARY AND OUTLOOK

The kinetoplastid protozoa possess protein-trafficking mechanisms that are in most aspects very similar to those of higher eucaryotes, including mammals and yeasts. Although—being diploid—kinetoplastids are not as experimentally manipulable as yeasts, they nevertheless have a role to play in the elucidation of protein trafficking mechanisms, in particular in highlighting universally conserved aspects. However, they are at their most interesting, from all points of view, when they differ from other cell types. Subtle differences in signal sequence specificities exist for all targeting sites so far examined—microbodies, mitochondria, and endoplasmic reticulum. Some components may to be missing in most (if not all) forms: clathrin-coated vesicles and recycling receptors. Other aspects are weird or unexplained: export of the gene B protein, the uptake mechanism of the transferrin receptor, mitochondrial import of cytochrome c_1, import of lysosomal matrix proteins, and the function of the cytostome. Some of these peculiarities may eventually prove to be weak points that can be used as targets for chemotherapy; others may, like RNA editing, turn out to be much more widespread than suspected. In any event, there are still plenty of unanswered questions.

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